AD_____

Award Number: W81XWH-06-1-0460

TITLE: The Role of ADAM9 in Tumor-Stromal Interactions in Breast Cancer

PRINCIPAL INVESTIGATOR: Jessica L. Fry

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

REPORT DATE: April 2008

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-04-2008	Annual Summary	31 Mar 2007 – 30 Mar 2008

4. TITLE AND SUBTITLE

The Role of ADAM9 in Tumor-Stromal Interactions in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0460

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jessica L. Fry

E-Mail: jessica_fry@student.hms.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Beth Israel Deaconess Medical Center
Boston, MA 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The tasks outlined in the Statement of Work for the second year of research on the action of ADAM9 isoforms in tumor-stromal interactions focused on the role of endogenous ADAM9 in breast cancer cell migration, and the elucidation of which major function of the protein is mechanistically relevant to cell migration. The major developments during this research period were the creation of an shRNA model system to evaluate the silencing of endogenous ADAM9 isoforms in breast cancer cell lines, and the reconstitution of gene expression with wild-type or functional mutants of both ADAM9 isforms to evaluate the distinct roles the functions of ADAM9 play in mediating cell migration.

15. SUBJECT TERMS
ADAM9, Tumor-Stromal Interactions, Breast Cancer, metastasis, migration

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction

The purpose of this research project is to evaluate the molecular mechanisms by which the isoforms of ADAM9 participate in breast cancer progression. Based on previous work, we hypothesized that ADAM9 participates in tumor-stromal interactions in breast cancer, acting to enhance tumor progression by mediating HB-EGF shedding and EGFR signaling, and participating in the epithelial-mesenchymal transition (EMT) through its integrin binding function. Our 2007 annual summary detailed the development of isoform specific ADAM9 antibodies and the specific detection of ADAM9 isoforms in breast cancer cell lines, as well as preliminary data identifying a role for ADAM9 in breast cancer cell migration. Recent literature has elucidated multiple candidate mechanisms by which the isoforms of ADAM9 participate in cell migration in addition to our original hypothesis of EGFR signaling, necessitating a broader approach than that described in the original statement of work. Using shRNA to silence endogenous ADAM9 and gene reconstitution experiments with both wildtype ADAM9 isoforms and functional mutants has given us valuable clues to the underlying mechanism by which ADAM9 is influencing cell migration. The results of this research will provide us with a new mechanism by which the ADAM9 isoforms present in breast tumors and stroma influence cell migration, lending insight into tumor progression and metastasis. Increased understanding of these mechanisms will lead to new strategies for therapy targeted specifically to advanced stage tumors. This research is also designed to broaden the knowledge, skills, and experience of the Principal Investigator and to provide the foundation for her career in breast cancer research.

Body

The following tasks from the Statement of Work for this project were the focus for the research period from 31 March 2007- 31 March 2008:

Task 1: To characterize the expression of ADAM9 in breast cancer tissues and cell lines representing different stages of the disease (months 1-13).

Task 2: Determine the role of ADAM9 in HB-EGF shedding and EGFR signaling in breast carcinoma cells (months 7-26)

The body of this summary will evaluate the progress made on each of these tasks, problems encountered, and immediate future directions.

Task 1:

The goal of this Task is to develop immunohistochemical tests to determine the presence of ADAM9 in breast cancer cell lines and tissue arrays, and to ultimately evaluate the isoform distribution in the tumors and stroma of breast cancers with different clinical profiles. Year one progress in this task included the development and testing of isoform-specific antibodies to both ADAM9-L and ADAM9-S, and the identification of breast cancer cell lines that endogenously express ADAM9-L and -S. Current research in the scope of this Task is dedicated to development of protocols to evaluate the efficacy and specificity of isoform specific antibodies in fixed-cell assays such as immunofluorescence and immunohistochemistry. I am currently visualizing endogenous ADAM9-L and ADAM9-S in BT549 breast cancer cell lines with and without silenced ADAM9 to evaluate the antibodies for immunofluorescence and verifying their use for subcellular localization studies. Immediate future work on this Task will be to evaluate the antibody in the immunocytochemical process, progressing to tissue array experiments to evaluate the localization of endogenous ADAM9 in an *in vivo* disease setting.

Current Data: Preliminary experiments indicate that the ADAM9L and ADAM9-S antibodies are successful in immunofluorescence in cells overexpressing ADAM9 isoforms. To move into tissue arrays, a protocol is being developed to detect endogenous levels of ADAM9 in breast cancer cell lines. Silencing ADAM-9 in BT549 cells resulted in only minor loss of immunofluorescence signal, despite loss of ADAM9 activity detected by immunoblot, and while there was very little background in cells transfected with ADAM-9 constructs (where only ~20% of the cells expressed ADAM-9 at all), cells endogenously expressing ADAM-9 have a high background level of fluorescence using my initial protocol. I am currently adjusting antibody dilutions and blocking solutions to eliminate this problem.

Summary and Current Work:

Technical difficulties in this section of the statement of work are being overcome by experimentation with various immunofluorescence protocols, antibodies (where they are commercially available), and ADAM9-expressing cell lines. Validating endogenous expression data *in vivo* using tissue arrays is the anticipated next step. There is no current data on the localization, specificity, or disease severity correlation of the different ADAM9 isoforms in human breast cancer tissues and we will explore these possibilities in the forthcoming year.

Task 2:

The goal of Task 2 is to evaluate the phenotypic contribution of ADAM-9 to the migration of breast cancer cells, with an emphasis on the cleavage of EGFR-ligands by ADAM-9. My original hypothesis,

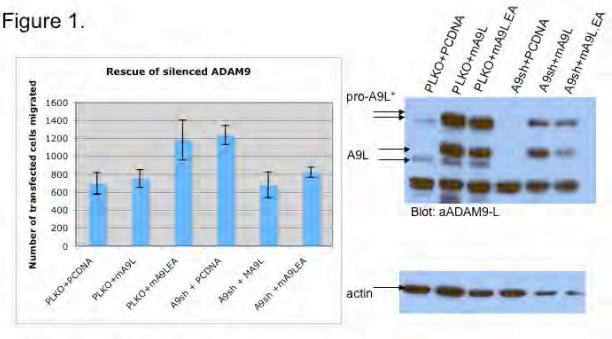
based on data from our lab in which both recombinant stimulation with and transfected and expressed ADAM9-S enhances invasion of breast cancer cells through a matrigel-coated transwell[1], was that overexpression of ADAM9-S in breast cancer cells would enhance migration, and that overexpression of ADAM9-L would either replicate this phenotype, or would have no migratory effect or an inverse migratory effect, indicating that the secreted form of ADAM9 was escaping regulation normally imparted through the cytoplasmic domain. Initial results, documented in Annual Summary 2007, indicated that overexpressing ADAM9-S in SUM159-PT cells did not enhance cell migration in a non-coated transwell, while a construct deficient in metalloprotease activity significantly enhanced migration in this assay. Later work showed that ADAM9-L overexpression increased migration in the same assay, which is abrogated by loss of the metalloprotease activity. Given that overexpression floods the model with the protein of interest, emphasis was placed on obtaining lentiviral vectors for the silencing of endogenous ADAM9 in the cell lines we identified as containing significant amounts of endogenous ADAM9, primarily the BT549 breast cancer cell line. As this task encompasses a large compilation of data, current work accompanies each section.

Current Data:

Lentivirally-infected silencing constructs for ADAM9 silence ADAM9 and enhance migration in a transwell assay.

Transfection of the Dharmacon siRNA into BT549 cells enhanced migration in an overnight transwell assay. Lentiviral constructs were made using the PLKO.1 vector and sequences obtained from Dharmacon and the MissionSiRNA project. Virus was made using HEK293T cells, and target BT549 cells were infected for 24 hours. After 24 hour selection (complete in comparison to non-infected controls) cells were transfected with beta-galactosidase. The next day, they were counted, and placed in the upper chamber of a 24-well transwell chamber, and allowed to migrate overnight (~16 hours). Cells were fixed and stained with beta-gal, and migrated transfected cells were counted manually under the microscope. In confirmation with the Dharmacon results, silencing of ADAM9 in BT549 cells results in an increase in their migration in this assay. This result has been replicated multiple times, individually and in concert with rescue experiments (discussed in the subsequent section) and representative data is available in Figure 1 (compare PLKO+PCDNA and A9shRNA+PCDNA data). Wound-healing experiments, in which a line is scratched in a monolayer of cells and the area of migration is calculated based on time to wound closure shows no difference

Figure 1.



Silencing of ADAM9 results in an increase in BT549 migration.
Rescue of lentiviral ADAM9 silencing with murine wt and metalloprotease deficient constructs results in rescue of the enhanced migration phenotype
*note: murine A9L is 30AA larger and runs higher than endogenous human ADAM9L

between silenced and non-silenced cells, indicating that cells are not simply migrating faster, but that they have a higher response to a chemoattractant gradient (data not shown). Given that overexpression of ADAM9-L enhanced, and overexpression of ADAM9-S had no effect on SUM159PT cell migration (a cell line with low endogenous ADAM9 expression), we are conducting gene reconstitution experiments using non-silenceable ADAM9-L and –S, as well as mutants of each to begin investigating the role of the metalloprotease and integrin-binding functions of ADAM9 isoforms in mediating migration.

Enhanced migration of BT549 cells in response to ADAM9 silencing is rescued by a murine ADAM9-L construct, as well as a metalloprotease-deficient ADAM9-L construct.

BT549 cells were infected as described above with the lentiviral sequence that most completely silences ADAM9 by immunoblot. Post-selection transfection with beta-gal was accompanied by transfection with PCDNA4 empty vector, murine ADAM9-L or murine ADAM9-L EA (a metalloprotease deficient construct). Cells were assayed in a transwell as described previously. Figure 1 shows that in addition to the expected increase in migration seen in ADAM9-silenced cells transfected with vector alone, the overexpression of murine ADAM9L.EA results in a marked increase in migration. This correlates with Paola Zigrino's recently published work, which shows enhanced keratinocyte migration on plates coated with recombinant cysteine-rich/disintegrin domains of ADAM9[2]. It also correlates with my previous data indicating that overexpressing the metalloprotease-deficient ADAM9-S construct increases migration in SUM159-PT cells, and together these data imply that metalloprotease-deficient ADAM9 enhances cell migration – conversely, that the metalloprotease activity of ADAM9 contributes to the migratory state of cells expressing endogenous ADAM9. As expected, the increase in migration seen when endogenous ADAM9 is silenced and "rescued" with an empty vector (PCDNA) is rescued with the addition of wild-type ADAM9-L (shA9 + mA9L). Reconstitution with the metalloprotease-deficient ADAM9L mutant also rescued the phenotype, indicating that it plays only a partial role. Construction of mutants in other functional domains, such as the disintegrin (integrin-binding), the cysteine-rich, PKCδ phosphorylation, and cytoplasmic domains are in the process of being constructed and evaluated to identify the key functional domains required for ADAM9 to mediate cell migration, in order to form an accurate model of the function of ADAM9.

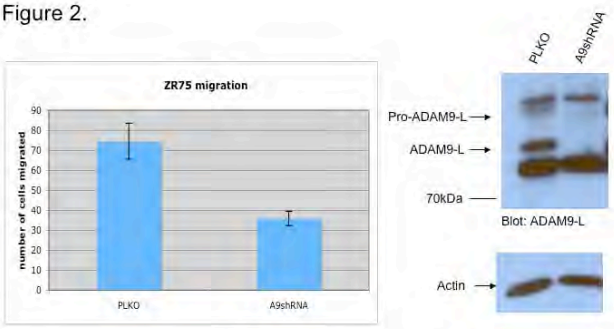
Current Work

1. Continue rescue experiments in BT549 cells using murine ADAM9L.EA, ADAM9.S, ADAM9S.EA, and mutants with deleted integrin binding/cysteine-rich/phosphorylation domains.

Silencing of ADAM9 decreases the migration of ZR75-1 breast cancer cells

In order to validate the migratory phenotype of silencing ADAM9 in multiple breast cancer cell types that express ADAM9, ZR75-1 cells, which have an amplified ADAM9 gene and express ADAM9-L to levels visible by western blot, were lentivirally infected with ADAM9 silencing constructs, selected, and then allowed to migrate through an uncoated transwell membrane. These cells are then stained with crystal violet, and the numbers of migrated cells are counted under the microscope. As a seeding control (to replace the beta-galactosidase transfection efficiency control in my previous experiments) I seeded equal numbers of cells in 2 wells per condition for the duration of the migration, and trypsinized and counted them and normalized my migration results to this number. Figure 2 shows representative data from multiple experiments, in which ZR75-1 migration is decreased upon silencing of ADAM9. It is important to note that in experiments conducted using identical conditions, BT549 cells continue to exhibit enhanced migration upon silencing of ADAM9. There are many known ways in which ZR75-cells differ from BT549 cells that can explain this phenotype difference, and may provide clues to the ultimate mechanism by which ADAM9 mediates cell migration. ZR75-1 cells belong to the luminal breast cancer gene cluster, while BT549 cells belong to the Basal B expression profile. In concert with this, in a 3D culture model, BT549 cells exhibit a stellate morphology, branching and invading the surrounding matrix, while ZR75-1 cells form grape-like clusters[3]. ZR75-1 cells also express much less β1 integrin in comparison to BT549 cells. β1 integrin has been shown to bind to ADAM9 and keratinocyte migration on an ADAM9 disintegrin/cysteinerich matrix is slowed by pre-incubation with a β1 integrin antibody[2]. In contrast to BT549 cells, ZR75-1 cells have not undergone EMT, as exhibited by their expression of E-Cadherin. The metalloprotease activity of ADAM9 has been shown to participate in the recycling of E-cadherin in HT29 colon cancer cells, preventing its degradation[4]. This opens an interesting hypothesis that ADAM9 strengthens adherens junctions in pre-EMT cells, lessening their migratory capacity, and functions in alternate pathways in cells that no longer express E-cadherin. In fact, N-Cadherin is known to potentiate mammary tumor metastasis via enhanced ERK signaling[5], a pathway that is also upregulated in cases of ADAM9 overexpression[2, 6]. Finally, BT549 cells express EGFR, while ZR75-1 cells do not, which is notable given that HB-EGF and EGF are reported substrates of ADAM9. To further evaluate the differences in ADAM-9 silencing in disparate cell types, we evaluated the expression of ADAM9, and the effects of silencing on migration in multiple cell types with different receptor and EMT profiles.

Figure 2.



ADAM9 silenced ZR75 cells exhibit a different migration phenotype than BT549s
ZR75-1 cells infected with ADAM9-siRNA exhibit a decrease in migration in a 6 hour migration assay. Cell numbers are representative of the average of 3 wells, 6 adjacent cell fields counted per well.

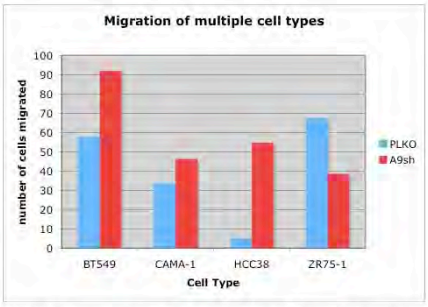
Current Work:

1. Evaluate the expression of ADAM-9 in BT549 and ZR75-1 like cell lines, and perform migration experiments on multiple cell lines with silenced ADAM-9 to determine the generality of the enhanced migration phenotype.
2. Develop retroviral ADAM-9 wild-type and functional mutant constructs to conduct rescue experiments in ZR75-1 cells due to their inability to transfect. This will ensure the results seen are a result of silencing ADAM-9 and not a phenotypic change due to another cellular process.

Evaluating the role of ADAM-9 in other ADAM-9 expressing cell types

Previous experiments using ZR75-1 and BT549 cells to evaluate ADAM9 in cell migration yielded an opposite phenotype upon silencing of ADAM9. It was determined that in addition to pursuing retroviral gene-replacement rescue experiments in ZR75-1 cells, evaluating more breast cancer cell lines for their response to ADAM-9 silencing would provide validation of either phenotype. Of the cell types that express ADAM-9 in the Neve et al. study[7], two cell types were chosen which are similar in phenotype to both BT549 and ZR75-1 cells. HCC38 is a Basal B gene cluster cell line that is ER/PR/HER2 negative and most resembles the BT549 cells in culture. CAMA-1 cells are luminal, ER/PR positive, and form grape-like structures in 3D culture similarly to ZR75-1 cells. ADAM9 is detected in each of these cell lines by Western blot, and migration studies were performed on these cells both in order to determine optimal incubation time, and in conditions where cells were lentivirally infected with ADAM9 silencing constructs, selected, and then allowed to migrate through an uncoated transwell membrane. These cells are then stained with crystal violet, and the numbers of migrated cells are counted under the microscope and data was normalized to a seeding control well to eliminate possible counting error. Preliminary results from this experiment show an increase in the migration of BT549, CAMA-1, and HCC38 through a transwell upon silencing of ADAM9, and ZR75-1 cells continue to show a decrease. These experiments are currently being repeated for accuracy, and upon conclusion these cell lines will be used in gene reconstitution experiments to confirm the generality of the ADAM-9 migration phenotype.

Figure 3.



ADAM9 silencing influences migration in a number of breast cancer cell lines. Preliminary data --BT549, CAMA-1, HCC38, and ZR75-1 breast cancer cell lines were lentivirally infected with vector control or shRNA to ADAM9. After 24 hour selection, cells were migrated 6 hours (with the exception of CAMA-1, for 24 hours) in a transwell assay, and counted after staining with crystal violet.

Current Work:

1. Rescue the migration phenotype in HCC38s and CAMA-1 cells with mutant and wild-type ADAM-9 constructs
2. Overexpress ADAM9 functional mutant constructs in cells that do not express high-levels of endogenous ADAM9 to compare to cells that do express ADAM9 endogenously.

Key Research Accomplishments

- The BT549 breast cancer cell line exhibits enhanced migration upon the silencing of ADAM-9. This enhancement is eliminated when exogenous ADAM9-L is introduced into the silenced cell, confirming the specificity of this phenotype.
- Reconstitution of ADAM9-L signaling in BT549 cells by introduction of a metalloprotease-deficient ADAM9L.EA construct rescues the enhanced migration phenotype in silenced BT549s.
- Enhancement of migration is preliminarily seen in multiple breast cancer cell types upon ADAM-9 silencing.
- The ZR75-1 breast cancer cell line exhibits decreased migration upon silencing of ADAM-9. This result is repeatable, but the gene reconstitution experiment has not yet been done.

Reportable Outcomes

- Invited Presentation: Era of Hope Meeting, June 2008

Conclusion

This second annual summary encompasses work done on Tasks 1 and 2 of the Statement of Work for this project. The development of a lentiviral shRNA system for silencing ADAM9 allows us to silence endogenous ADAM-9 in human breast cancer cell lines, and to identify the migration phenotype. Combined results over two years seem to indicate that ADAM9 suppresses migration of breast cancer cells. Reconstitution experiments using wild-type ADAM9-L eliminate this phenotype, while very preliminary results show that ADAM9-S enhances migration in both the presence and absence of both ADAM9 isoforms, which is analogous to what was observed in overexpression experiments catalogued in our 2007 annual summary. This, in addition to the opposing phenotype seen when silencing ADAM9 isoforms in ZR75-1 breast cancer cells, indicates that the role of ADAM9 in migration is complex, and that the membrane-bound and secreted isoforms may be acting selectively through different pathways, and may be genotype specific. This is especially important given the identification of ADAM9 as a potential target of breast cancer therapy [7]. Studies that consider the functional role of each isoform in different genotypic contexts, *in vitro* as well as *in vivo*,

will be important for the development of safe and effective treatments. Our work with the lentiviral shRNA system and gene reconstitution experiments has lent insight into the putative function of ADAM9 through its integrin-binding domain, and our future work in this area will evaluate the functional domains of each ADAM9 isoform to define the relevant signaling pathways which participate in ADAM9 mediated cell migration, paving the way for future studies into the role of ADAM9 isoforms in breast cancer invasion and metastasis.

References:

1. Mazzocca, A., et al., *A secreted form of ADAM9 promotes carcinoma invasion through tumor-stromal interactions.* Cancer Res, 2005. **65**(11): p. 4728-38.
2. Zigrino, P., et al., *Role of ADAM-9 disintegrin-cysteine-rich domains in human keratinocyte migration.* J Biol Chem, 2007. **282**(42): p. 30785-93.
3. Lee, G.Y., et al., *Three-dimensional culture models of normal and malignant breast epithelial cells.* Nat Methods, 2007. **4**(4): p. 359-65.
4. Hirao, T., et al., *Overexpression of ADAM9 enhances growth factor-mediated recycling of E-cadherin in human colon cancer cell line HT29 cells*. Exp Cell Res, 2006. **312**(3): p. 331-9.
5. Hulit, J., et al., *N-cadherin signaling potentiates mammary tumor metastasis via enhanced extracellular signal-regulated kinase activation.* Cancer Res, 2007. **67**(7): p. 3106-16.
6. Karadag, A., M. Zhou, and P.I. Croucher, *ADAM-9 (MDC-9/meltrin-gamma), a member of the a disintegrin and metalloproteinase family, regulates myeloma-cell-induced interleukin-6 production in osteoblasts by direct interaction with the alpha(v)beta5 integrin*. Blood, 2006. **107**(8): p. 3271-8.
7. Neve, R.M., et al., *A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes.* Cancer Cell, 2006. **10**(6): p. 515-27.